In the present study we utilized a semisolid culture system with feeder cells and enriched media to evaluate the growth of acute leukemia associated with the 4;11 chromosomal translocation. We compared growth of t(4;11) leukemia to typical acute nonlymphocytic leukemia (ANL) and acute lymphocytic leukemia (ALL). The two cases of t(4;11) leukemia tested exhibited the highest cloning efficiency of cells tested. The growth characteristics of t(4;11) leukemia were more similar to ANL than ALL.

By Robin C. Stong and John H. Kersey

MUCH EFFORT in recent years has been directed at elucidation of the heterogeneity of the acute leukemias of humans. Morphological, cytochemical, chromosomal, and cell surface phenotype studies have advanced our understanding of these leukemias.1,2 Similarly, studies of leukemic cells in semisolid culture systems have been of value. Such culture systems have frequently used feeder cells and/or enriched conditioned media.3 With the use of these conditions, growth of cells from patients with typical acute nonlymphocytic leukemia (ANL) is quite reproducible.4,5 Successful cultures from patients with typical acute lymphoblastic leukemia (ALL) are more difficult to obtain, but such cultures are sometimes possible.6,7

Acute leukemia with the 4;11 chromosomal translocation, generally termed t(4;11) ALL, is an uncommon but important form of acute leukemia in that patients have a very poor prognosis8 and that in the leukemic cells exhibit phenotypic features of both lymphoid and myeloid cells.9 In the present study, we compared the growth characteristics of acute leukemias and demonstrated that t(4;11) cells grow in culture more like ANL than ALL cells.

MATERIALS AND METHODS

Patients. Bone marrow samples were collected by routine procedures from 11 patients with acute leukemia. Five of these patients were in relapse at the time of study, and four of these had relapsed while on chemotherapy. At the time of aspiration, patient 4 was receiving dihydroxyaztrencene; patient 5 was receiving 6-mercaptopurine, methotrexate, vincristine, and predniocrine; patient 6 was receiving 6-mercaptopurine and methotrexate; and patient 8 was receiving indocine-N-oxide. Diagnosis was based upon the morphology of Wright-stained blast cells, ultrastructural characteristics, cytochemical staining (myeloperoxidase, Sudan black B, periodic-acid Schiff, and nonspecific esterase), and analysis of surface membrane markers. Receptors for sheep erythrocytes were detected using a standard rosetting technique.10 Fc receptors were detected by direct immunofluorescence assay, using heat-aggregated IgG.11 Cell surface immunoglobulin was assayed by direct immunofluorescence with fluorescein-isothiocyanate (FITC)-conjugated goat anti-human total immunoglobulins (Kallestad, Austin, Tex.). Terminal deoxynucleotidyl transferase (TdT) was detected by immunofluorescence assay as previously described.12 An indirect immunofluorescence test was used to evaluate reactivity with monoclonal antibodies (MoAb) directed against the following specificities: HLA-DR (Becton Dickinson, Sunnyvale, Calif); [p95, 170] TA-1, which is expressed on most T cells and monocytes13; the T cell-specific markers CD2 [T, p50] 35.1,15 CD3 [T, p19, 29] OKT3,14 CD1 [Thy, p45-12] OKT616 (Ortho Pharmaceutical Corp, Raritan, NJ), the myelomonocytic markers CD11 [M, G, p150, 95] OKM117 (Ortho) and CD14 [M, G, U] M-CSF;18 and the B lineage-associated markers CD10 common acute lymphocytic leukemia antigen [CALLA] BA-319 CD24 [p45, 55, 65] BA-1,20 and CD9 [nT-nB, p24] BA-2.1 The [p95, 170] TA-1 determinant is expressed in all types of ANL, but is most frequently expressed in monocytic leukemias of M4 and M5 type.12 The MoAb cluster designations are those assigned at the First and Second International Workshops on Human Leukocyte Differentiation Antigens.19 Control asctic fluid substituted for monoclonal antibody was used as a control for nonspecific binding. Monoclonal antibodies were used in antibody excess. FITC-conjugated goat F(ab')2, anti-mouse IgG (Cappel Laboratories, Cochranville, Penn.) was used as second antibody. Cells were evaluated with a Zeiss fluorescent microscope equipped with Ploem eill-illumination. In some cases, sample size limited the numbers of markers assayed.

Karyotype analyses were performed on G-banded preparations as previously described.20,21 All procedures were approved by the Committee for Human Subjects in Research at the University of Minnesota. Informed consent was obtained from the patient or from the parents. Patient data are summarized in Table I.

Sample processing. Mononuclear cells were obtained by Ficoll-Hypaque density gradient centrifugation.22 Interface cells (density 1.077 g/cm³) were collected, washed, and mixed with 2.5-aminomethylisothioauronium (AET)-treated sheep erythrocytes (SRBC) for 15 minutes at 37 °C and then incubated for one hour at 4 °C. The rosette-forming T lymphocytes were removed by a second Ficoll-Hypaque centrifugation. Depleted cell populations were found to contain fewer than 2% T cells based on their reactivity with the pan-T cell MoAb 35.1 and OKT3. Washed cells were suspended at 4 x 10⁶ cells/mL in α-minimum essential medium (α-MEM) (KC Biologicals, Lenexa, Kan) with 20% fetal calf serum (FCS) (Reheis Chemical Co, Kankakee, Ill), 10 IU penicillin/mL and 100 µg streptomycin/mL (hereafter referred to as growth medium). Cells were incubated at 37 °C in plastic tissue culture flasks for two to 14 hours to deplete adherent cells. Cytosin preparations of the enriched populations were stained with Wright's-Giemsa. The leukemic cell-enriched population was then counted and plated in the methylcellulose colony assay.

Feeder cells and conditioned medium. Peripheral blood T lymphocytes from normal donors were used as feeder cells and to prepare conditioned medium. Mononuclear cells were obtained following Ficoll-Hypaque centrifugation, mixed with AET-treated SRBC as described above, and T rosettes were recovered from the pellet after a second Ficoll-Hypaque centrifugation. The SRBC were lysed with ammonia-chloride Tris buffer to produce essentially pure T cell populations.

Conditioned medium was prepared by culturing T cells (1 x 10⁶/mL) with 1% phytohemagglutinin (HA-17, Burrroughs Wellcome, Research Triangle Park, NC) in growth medium. The

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Supported in part by grants No. CA25097 and CA21737 from the National Cancer Institute, National Institutes of Health.
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0006-4971/85/6602-0010$03.00/0

cultures were maintained at 37 °C for three days in a humidified atmosphere of 5% CO₂/95% ambient air. Culture supernatant was collected, centrifuged, filter sterilized, and stored at 4 °C. A pool of T cell conditioned media (PHA-TCM) prepared from three separate donors was used in these experiments.

Feeder cells were prepared from freshly isolated T cells. These were incubated at 37 °C for two hours, placed in ice, and then irradiated (2,000 rad) by a Mark IV cesium irradiator.

**Colony assay.** Leukemic cell-enriched populations were cultured using the system described by Izaguirre et al. In brief, leukemic cells (1 to 4 x 10⁵/mL) and feeder cells (3 x 10⁵/mL) were mixed with 0.8% methylcellulose, 10% to 30% PHA-TCM, and growth medium. Cell mixtures were dispensed into 96-well flat-bottom tissue culture plates (Linbro, Flow Laboratories, McLean, Va) and cultured at 37 °C in an incubator chamber (Billups-Rothenberg, Del Mar, Calif) that had been flushed with a mixture of 5% O₂, 5% CO₂, and 90% N₂. Chambers were flushed daily. In each experiment, cultures containing all components except leukemic cells were plated as a control to ensure that irradiated T cell feeders did not form colonies.

**Colony evaluation.** After seven to 13 days of incubation, cultures were evaluated using an inverted phase-contrast microscope, and groups of 20 or more cells were scored as colonies. Individual colonies were aspirated with a finely drawn Pasteur pipette. Cells from 50 to 100 colonies were pooled and washed. Cytospin preparations were stained with Wright’s-Giemsa, and the morphology of colony cells was compared to that of the preculture population. Remaining colony cells were evaluated for reactivity with BA-1, BA-2, BA-3, 35.1, MCS, anti-HLA-DR, and anti-TdT as cell numbers permitted; 50 to 100 cells were scored for each marker.

**RESULTS**

Bone marrow samples from all 11 leukemic patients were cultured in our methylcellulose colony assay system. As shown in Table 2, five of these samples grew colonies of >20 cells (patients 1 through 5); the cultures from the remaining six patients (all with ALL) showed no growth.

Prior to culture, blasts from patient 1 with AML (French-American-British classification M2; FAB M2) were positive for myeloperoxidase, and rare cells contained Auer rods. Nonspecific esterase staining was negative. Compact, nonadherent leukemia, also gave rise to colonies in our culture system. The colonies were compact, nonadherent and nearly spherical, each containing 20 to 40 cells by day 11. The cloning efficiency was 0.24%. Analysis on day 12 revealed lymphoblast appearing cells that resembled those seen prior to culture (Fig 1). These cells were strongly TdT-positive and

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Diagnosis</th>
<th>New Case or Relapse</th>
<th>Sex/Age (yr)</th>
<th>Bone Marrow (Percentage of Blasts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AML</td>
<td>Relapse</td>
<td>M/10</td>
<td>82.2</td>
</tr>
<tr>
<td>2</td>
<td>AML</td>
<td>New</td>
<td>M/17</td>
<td>96.0</td>
</tr>
<tr>
<td>3</td>
<td>AML</td>
<td>New</td>
<td>F/3</td>
<td>83.0</td>
</tr>
<tr>
<td>4</td>
<td>AL</td>
<td>Relapse</td>
<td>F/1</td>
<td>97.0</td>
</tr>
<tr>
<td>5</td>
<td>AL</td>
<td>Relapse</td>
<td>F/32</td>
<td>98.5</td>
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<tr>
<td>6</td>
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<td>Relapse</td>
<td>M/6</td>
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<td>ALL</td>
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</tr>
<tr>
<td>8</td>
<td>ALL</td>
<td>Relapse</td>
<td>M/8</td>
<td>90.0</td>
</tr>
<tr>
<td>9</td>
<td>ALL</td>
<td>New</td>
<td>M/5</td>
<td>90.0</td>
</tr>
<tr>
<td>10</td>
<td>ALL</td>
<td>New</td>
<td>F/3</td>
<td>97.8</td>
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<tr>
<td>11</td>
<td>ALL</td>
<td>New</td>
<td>M/38</td>
<td>95.0</td>
</tr>
</tbody>
</table>

AML, acute myeloid leukemia; AML, acute megakaryocytic leukemia; AL, acute leukemia; ALL, acute lymphocytic leukemia.

Bone marrow from patient 2, whose AML (FAB:M1) blasts stained with Sudan black B and myeloperoxidase prior to culture, grew small colonies with a cloning efficiency of 0.02%. At day 8, the colonies were composed of approximately 20 round, tightly clustered cells that rested on two to four intertwining, elongated, and adherent cells. Colonies were isolated, with care being taken to recover both elongated and round cells within the colony. Pooled colony cells reacted strongly with the myeloid antibody MCS (Table 2). The morphology of these cultured cells in Wright-Giemsa-stained preparations was identical to that seen with fresh patient’s cells.

Patient 3 with acute megakaryocytic leukemia had leukemic blasts characterized by large and predominantly multinucleated nuclei. Ultrastructurally, the large blasts contained clusters of bull’s-eye granules similar to those present in megakaryocytes and platelets. Bone marrow from patient 3 formed rare colonies that were composed of adherent, elongated cells with 20 to 25 small round cells clustered on top. Isolated adherent cells that had fibroblast morphology were common in the cultures. Too few colonies were recovered from the methylcellulose medium to allow characterization of the colony cells.

Successful cultures were obtained from the two patients with t(4;11) leukemia who were tested. In both cases, >90% of blasts were strongly positive for TdT prior to culture. For patient 4, 20-cell colonies were apparent by day 5. Each colony consisted of four to ten elongated, adherent cells with a tight cluster of ten to 15 round, highly refractile cells resting on top. The number of round, tightly associated cells in the clusters increased to a maximum of about 30 cells on day 9. Colony counts performed on day 8 revealed an average of 122.5 colonies per well, giving a cloning efficiency of 0.31%. Cells from pooled colonies had the appearance of lymphoblasts and were morphologically identical to preculture cells from the patient. TdT staining was not performed on colony cells. The vast majority of cells strongly expressed the CD24 [p45, 55, 65] BA-1 and the CD9 [p24] BA-2 antigens, but did not react with CD10 [CALLA] BA-3 reagent nor with the CD2 [T, p50] 35.1 reagent. Thus, their phenotype is consistent with that of preculture lymphoblasts (Table 2).

The leukemic cells from patient 5, an adult with t(4;11)-associated leukemia, also gave rise to colonies in our culture system. The colonies were compact, nonadherent and nearly spherical, each containing 20 to 40 cells by day 11. The cloning efficiency was 0.24%. Analysis on day 12 revealed lymphoblast-appearing cells that resembled those seen prior to culture (Fig 1). These cells were strongly TdT-positive and
Table 2. Frequency of Leukemic Cell Colonies in Methylenblue Culture and Phenotypic Comparison of Colony Cells to Preculture Patient Bone Marrow

<table>
<thead>
<tr>
<th>Case No.</th>
<th>No. of Cells Plated</th>
<th>No. of Colonies</th>
<th>Cloning Efficiency (%)</th>
<th>CD24/BA1</th>
<th>CD29/BA2</th>
<th>CD10/BA3</th>
<th>CD2/35.1</th>
<th>CDw13/MCS2</th>
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<tbody>
<tr>
<td>1</td>
<td>6 x 10⁴</td>
<td>9.5 ± 0.7</td>
<td>.020</td>
<td>4.5</td>
<td>4</td>
<td>2.0</td>
<td>10.0</td>
<td>49.0</td>
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<tr>
<td>2</td>
<td>6 x 10⁴</td>
<td>12.0 ± 5.6</td>
<td>.020</td>
<td>34.0</td>
<td>69</td>
<td>0.5</td>
<td>19.0</td>
<td>NT</td>
</tr>
<tr>
<td>3</td>
<td>4 x 10⁴</td>
<td>0.4 ± 0.5</td>
<td>.001</td>
<td>87.0</td>
<td>37</td>
<td>0</td>
<td>0</td>
<td>76.0</td>
</tr>
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<td>4 x 10⁴</td>
<td>122.5 ± 10.6</td>
<td>.310</td>
<td>62.0</td>
<td>92</td>
<td>0</td>
<td>4.0</td>
<td>NT</td>
</tr>
<tr>
<td>5</td>
<td>4 x 10⁴</td>
<td>97.0 ± 19.0</td>
<td>.240</td>
<td>20.0</td>
<td>86</td>
<td>0</td>
<td>1.0</td>
<td>9.5</td>
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<tr>
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<td>4 x 10⁴</td>
<td>0</td>
<td>0</td>
<td>72.0</td>
<td>3.5</td>
<td>52.5</td>
<td>4.0</td>
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<td>0</td>
<td>86.5</td>
<td>66.5</td>
<td>92.5</td>
<td>2.5</td>
<td>7.0</td>
</tr>
<tr>
<td>8</td>
<td>6 x 10⁴</td>
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<td>0</td>
<td>67.0</td>
<td>30.0</td>
<td>90.5</td>
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<td>1.0</td>
</tr>
<tr>
<td>9</td>
<td>4 x 10⁴</td>
<td>0</td>
<td>0</td>
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<td>46.0</td>
<td>84.0</td>
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<td>0</td>
<td>87.5</td>
<td>77.0</td>
<td>85.0</td>
<td>5.0</td>
<td>7.0</td>
</tr>
<tr>
<td>11</td>
<td>4 x 10⁴</td>
<td>0</td>
<td>0</td>
<td>73.0</td>
<td>54.0</td>
<td>61.5</td>
<td>3.5</td>
<td>2.0</td>
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</tbody>
</table>

*Numbers represent the percentage of cells reactive with MoAb.
expressed CD9 [p24] BA-2. They did not express [CALLA] BA-3, T cell markers, nor the myelomonocytic markers CDwI3/MCS2. The expression of CD24[p45, 55] BA-1 and HLA-DR was greater on colony cells than on the uncultured blast population; CD24-positive cells increased from 20% to 74% and HLA-DR positive cells increased from 30% to 84% during culture.

In experiments with bone marrow from patient 5, duplicate plates were cultured in a standard incubator containing 5% CO2 in air (O2 concentration 18% to 20%). Small clusters containing four to ten cells formed, but by day 5 these cells had died, based on trypan blue exclusion. This finding suggests the importance of low O2 concentration for colony formation by t(4;11) leukemic cells.

The six samples obtained from patients with CALLA-positive acute lymphocytic leukemia grew only small clusters containing four to ten cells in our culture system. Too few cells were recovered from these clusters to test for membrane markers. It should be noted that the culture reagents used for these experiments with ALL were the same as those that gave successful colony growth with ANL and t(4;11) AL. Conditioned medium prepared from several different T cell donors as well as conditioned medium prepared by stimulating human spleen cells with pokeweed mitogen were also tested for ability to support colony growth by ALL cells; all were without effect.

Karyotypes were performed on nine of the 11 bone marrow samples used in this study. Although no consistent chromosomal abnormality was associated with the ability to form colonies in methylcellulose medium, the finding that both cases of t(4;11) leukemia and all cases of ANL formed colonies appears to be significant. The data summarized in Table 1 indicate that colony formation did not correlate with age or sex of the patient or with the percentage of blasts.

**DISCUSSION**

The results of this study demonstrate that acute leukemia with the translocation involving chromosomes 4 and 11 requires distinctive culture conditions for clonogenic growth. To our knowledge, these studies are the first in which the culture characteristics of t(4;11) cells have been reported. Notably, we found cell growth requirements of t(4;11) leukemia to be similar to those of acute nonlymphocytic leukemia rather than those of acute lymphocytic leukemia. These results are of interest because t(4;11) acute leukemia has traditionally been classified as a form of ALL based primarily on the morphological appearance of the leukemic cells.

Our results support previous reports that t(4;11) acute leukemia has characteristics that are distinctive from common ALL and which in part are myeloid in nature. These previous studies5,8 have demonstrated that t(4;11) leukemia cells exhibit both lymphoid and myeloid phenotypic markers and lack the common ALL antigen. Some cells exhibit basophil or mast cell morphology by light and electron microscopy.9 Cells from t(4;11) acute leukemia have also been shown to develop characteristics of monocytic cells, including phagocytosis, following incubation with phorbol esters.10 Recently we established a cell line from a patient with t(4;11) acute leukemia27 (patient 5 in the present series). This cell line is of interest in that the cells show clear evidence of commitment to lymphoid lineage, with immunoglobulin heavy and light chain gene rearrangement and with some cells expressing cytoplasmic immunoglobulin. Notably, these cells also exhibit myeloid characteristics; they bear the myeloid antigen CDw15, [G,CHO] IG10 and, after phorbol ester treatment, develop phagocytic function and express the monomyelocytic markers CD11/OKM1 and CDw13/MCS2. In the present study, colony cells from the t(4;11) AL patients did not exhibit monocytic morphology nor did they express the monomyelocytic marker CDw13; this indicates that the leukemic cells did not differentiate during culture.

The in vitro culture of cells from acute leukemia has been a subject of interest. Several groups have shown that acute nonlymphocytic leukemia cells may be grown using enriched media, serum, leukocyte-conditioned supernatants, feeder layers, and semisolid media.4-6 Slight variations of these culture conditions have supported the growth of cells from some patients with ALL, although such growth has been more difficult to attain.7,8 These previously reported results suggest that the growth factor requirements for ALL and ANL differ, although in both cases, the precise growth requirements remain poorly defined. Whatever the explanation for the difference in growth of ALL and ANL, our results are not unlike those of most other groups, and most important, they suggest that t(4;11) acute leukemia behaves more like ANL than ALL in culture. The cloning efficiencies
obtained with the t(4;11) leukemias were higher than those obtained with the myeloid leukemias of M1 and M2 FAB types. Because t(4;11) AL blasts exhibit some characteristics that are monocytic in nature, one wonders if M5-AML cells would give similarly high cloning efficiencies in our culture system.

Additional studies with more patients and different preparations of leukocyte conditioned media and/or feeder layers will be necessary to demonstrate the generality of our observations. Definition of the precise growth factor requirements for each of the major forms of acute leukemia will provide a long-term challenge; the goal to define these differences precisely should not detract from the observation that t(4;11) leukemia has growth requirements that are more similar to ANL than to ALL.

REFERENCES

In vitro culture of leukemic cells in t(4;11) acute leukemia

RC Stong and JH Kersey